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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2017.01.007

Expression and clinical significance of high risk human papillomavirus and invasive gene in cervical carcinoma

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ARTICLE INFO

Article history:
Received 12 Oct 2016
Received in revised form 24 Nov 2016
Accepted 10 Dec 2016
Available online 19 Jan 2017

Keywords:
Human papillomavirus
mRNA expression
Cervical cancer

ABSTRACT

Objective: To study the expression of E6 and E7 mRNA in high-risk human papillomavirus (HPV) HPV-18 and the relationship between the expression of invasive gene and cervical carcinoma.

Methods: A total of 119 patients with cervical cancer, cervical erosion and cervical HPV infection who were diagnosed in our hospital were selected and randomly divided into two groups: cervical cancer group ($n = 58$) and non-cancerous group ($n = 61$). Another 60 patients with uterine leiomyoma were selected as normal control group. Detection of HPV18 E6, E7 mRNA expression and invasion, migration, proliferation inhibition genes, epithelial mesenchymal transition genes and proliferation related protein content.

Results: The relative expression of E6 and E7 HPV-18 in cervical cancer group was significant higher than that in non-cancerous group and control group (mRNA) ($P < 0.05$). The content of TRAF6 and c-FLIP in invasive cervical cancer group was significantly higher than that in non-cancerous group and control group ($P < 0.05$). The mRNA content of CD44v6 and MMP-9 in cervical cancer group was significantly higher than that in non-cancerous group and control group ($P < 0.05$). The content of DEC-1, IKK16, MBP-1 in cervical cancer group was significant lower than that in non-cancerous group and control group ($P < 0.05$). The mRNA content of beta -catenin and Vimentin in cervical cancer group was significantly lower than that in non cancerous group and control group ($P < 0.05$). The proliferation related protein E2F1 of cervical cancer group was significantly lower than that of non-cancerous group and control group, Bmi-1 content was significantly higher than non-cancerous group and control group ($P < 0.05$).

Conclusions: The expression of the detection of cervical cancer in high-risk human papilloma virus HPV-18 E6 and E7 mRNA, and the invasion, migration, proliferation inhibition gene, epithelial mesenchymal transition and proliferation related gene protein content, HPV expression rate of mRNA increased with the development of cervical cancer, the expression is also enhanced. The expression has a certain correlation between the level and development of cervical cancer. Through the above indicators, the development of cervical cancer monitoring and treatment to provide important clinical guidance.

1. Introduction

Cervical carcinoma is the second most common malignant tumor only after breast cancer in women, which is the highest degree of malignancy. Nearly ten thousand women were diagnosed with cervical cancer worldwide each year, especially in developing countries and shows younger trend, of which the mortality ranks first in female malignancies. The global average annual new cervical carcinoma is more than 50 million cases, with death of over 20 million cases. In China, there are 130000 new cases each year, accounting for 1/3 of the world. In 1970s, since German virologists has presented the hypothesis that human papillomavirus (HPV) is closely related to the incidence of cervical cancer, numerous studies have shown that HPV infection, in particular high-risk HPV persistent infection, is the main

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Peer review under responsibility of Hainan Medical University.

reason for vast majority of cervical precancerous lesions and cervical carcinoma [1,2]. HPV infection is the leading pathogenic factor of cervical carcinoma. The occurrence of 99.7% cervical carcinoma is related to HPV infection, and the same subtype of high-risk HPV persistent infection is the leading cause of cervical carcinoma [3–6]. There are about 193 kinds of HPV DNA or mRNA detection methods in the world, and it is very important to check HPV for the diagnosis, the evolution of cervical cancer, the follow-up and prognosis evaluation. To discuss the HPV18 E6, E7 mRNA expression in cervical carcinoma tissue and its relationship with cervical carcinoma, to analyze its interaction with cervical carcinoma and to study the role and mechanism of HPV18 E6, E7 mRNA in cervical carcinoma will provide biomolecular evidence for the formation of cervical carcinoma, thus guiding early diagnosis, prevention and treatment.

2. Materials and methods

2.1. General materials

A total of 119 patients with cervical cancer, cervical erosion and cervical HPV infection who were diagnosed in our hospital from 1st February to 30th June 2015 were selected and randomly divided into two groups: cervical cancer group (n = 58) and non-cancerous group (n = 61). In cervical cancer group (n = 58), the age range was 36–60 years, as for CIN type, there were 23 cases of type I, 19 cases was type II, and 16 cases was type III. While in non-cancerous group (n = 61), the average age range was 34–57 years, 34 cases were cervical erosion and 27 cases were cervical HPV infection. Another 60 patients who underwent resection of uterine leiomyoma were enrolled as normal control group. Inclusion criteria: (1) patients associated with cervical hyperemia, erosion and cervical hyperplasia; (2) patients are in line with "TBS classification" in the diagnostic criteria revised by the International Cancer Society; (3) patients without sex hormones treatment or undergoing cervical surgery before admission within 3 months. Clinical manifestations were mainly diagnosed with contact bleeding or abnormal vaginal bleeding, abnormal vaginal discharge, lower abdominal pain. All the patients had no history of CIN, cervical cancer, pelvis radiation therapy, total hysterectomy and without pregnancy. Three days before collecting samples, patients did not have vaginal irrigation and drug use. Normal cervical tissue was obtained from the uterine cervix for excision of uterine leiomyoma. All of the patients were not treated with radiotherapy and chemotherapy before undergoing operation. All patients were operated by a skilled clinician. Part of the tissue was used for pathological diagnosis, and the remaining part was frozen in liquid nitrogen for the extraction of mRNA. Consents were obtained from the patients before the experiment and informed consents were signed. General materials like gender and age in each group had no statistical difference and were comparable ($P > 0.05$).

2.2. Methods

2.2.1. Collection and preservation of the clinical samples

The appropriate amount of specimens of the lesion was taken and cleaned by saline for 3–5 times. After that, sample was transferred into the cryopreservation tube, frozen in liquid nitrogen for 30 min, and placed in refrigerator at −80 °C for further use.

2.2.2. Determination of HPV18 E6 and E7 mRNA

Reverse Transcription System (Promeg cat# A 3500) kits were used and strictly performed according to the Kit instructions for reverse transcription. RNA sample of 9.75 μL (1 μg) was added, and after sufficient mixing of reaction system (20 μL), centrifugation was used. The reaction condition of the sample was: 42 °C, 60 min, 99 °C, after 5 min the reaction was ended, and the outcome was cooling at 4 °C and placed at −20 °C for further use. Primer sequence of gene HPV18 E6 was U: GCGCTTTGAGGATCCAACAC, D: ACGAATGGCACT GGCCTCTA, with the amplified length of 415 bp; Primer sequence of gene HPV18 E7 was U: AAGAAAACGAT-GAAATAGATGGA, D: GGCTTCACACTTACAACACA, with the amplified length of 100 bp. The PCR reaction system was as follows: 2.5 μL 10 × PCR buffer, 0.5 μL dNTPs (10 mmol/L), 25 mmol/L $MgCl_2$ 0.8 μL, 0.1 μL Taq enzyme (5 U/μL), primer F (10 μg/L) 0.5 mL, 0.5 μL primer R (10 μg/L), probe (10 μg/L) 1 μL, 5 μL cDNA, adding ddH_2O to 25 μL system. After centrifugation for 10 s at low temperature, PCR was carried out, with the apparatus selected of Roche Light Cycler fluorescence PCR Thermal cyclers. The reaction temperature transmission rate of each step was set to 20 °C/s, and each cycle of fluorescence was detected at 62 °C of 40 s. A negative control was used to monitor the experimental contamination. Positive control was used to monitor if RNA was extracted successfully. The ratio of E6, E7 expression and internal reference β-actin expression was regarded to its relative expression.

2.2.3. Detection method of the target gene

Tissues of cervical cancer, cervical erosion and cervical HPV infection were collected and Trizol lysate was added to grind the tissues sufficiently. Then RNA was reverse transcribed into cDNA using a reverse transcription kit. cDNA sample was taken to perform fluorescent quantitation PCR and amplified the target gene TRAF6, c-FLIP, D44v6, MMP9, DEC-1, IKK16, MBP-1, β-catenin, Vimentin, E2F1, Bmi-1 and internal reference gene GAPDH, respectively. After obtaining amplification curve, the mRNA contents of the above-mentioned genes were calculated with GAPDH as the internal reference.

Table 1
Comparison of HPV18 E6, E7 mRNA and invasion gene expression in each group.

Group	n	HPV-18 E6 mRNA	HPV-18 E7 mRNA	TRAF6	c-FLIP
Cancerous group	58	3.76 ± 0.31	2.89 ± 0.21	2.32 ± 0.31	2.17 ± 0.24
Non-cancerous group	61	0.20 ± 0.04*	0.19 ± 0.03*	0.61 ± 0.07*	0.59 ± 0.16*
Control group	60	0.24 ± 0.04*	0.23 ± 0.02*	0.46 ± 0.04*	0.44 ± 0.06*

($\bar{x} \pm s$).
Note: Compared with cancerous group, *$P < 0.05$.

2.3. Statistical analysis

SPSS16.0 statistical software was used to analyze the experimental data. *T*-test was used for measurement data which were expressed as $(\bar{x} \pm s)$. χ^2 was used for enumeration data. Spearman test was used for correlation analysis. $P < 0.05$ was considered as significant difference.

3. Results

3.1. Comparison of HPV18 E6, E7 mRNA and invasion gene expression in each group

In comparison of HPV18 E6, E7 mRNA and invasion gene expression in each group, E6 and E7 mRNA expression of HPV18 had no significant difference in non-cancerous group compared with those in control group ($P > 0.05$). E6 and E7 mRNA expression of HPV18 was all higher in cancerous group compared than those of in non-cancerous and control groups, which had significant difference ($P < 0.05$). The contents of TRAF6 and c-FLIP in non cancerous and control groups had no significant difference ($P > 0.05$). The contents of invasion genes TRAF6 and c-FLIP were all higher in cancerous group compared than those of in non cancerous and control groups, which all showed significant difference ($P < 0.05$) (Table 1).

3.2. Comparison of expression of migration and proliferation inhibitor gene in each group

The contents of migration genes CD44v6 and MMP9 mRNA had no significant difference in non cancerous and control groups ($P > 0.05$); the mRNA contents of CD44v6 and MMP9 in cancerous group was significant higher than those of in non cancerous and control groups, which all showed statistical difference ($P < 0.05$). The contents of proliferation inhibitor genes DEC-1, IKK16 and MBP-1 had no significant in the comparison of non cancerous and control groups ($P > 0.05$). In cancerous group, the contents of proliferation inhibitor genes DEC-1, IKK16 and MBP-1 were obviously lower than those of in non cancerous and control groups, which all showed statistical difference ($P < 0.05$) (Table 2).

3.3. Comparison of contents of epithelial and stromal transforming gene and proliferation related protein in each group

The mRNA contents of epithelial and stromal transforming genes β-catenin and Vimentin had no significant difference in non-cancerous and control groups ($P > 0.05$). In cancerous group, the mRNA contents of β-catenin and Vimentin were obviously lower than those of in non-cancerous and control groups, which all showed statistical difference ($P < 0.05$). The contents of proliferation related proteins E2F1 and Bmi-1 had no significant difference in non-cancerous and control groups ($P > 0.05$). In cancerous group, proliferation related protein E2F1 was significantly lower than that of in non-cancerous and control groups, while the content of Bmi-1 was obviously higher than that of in non-cancerous and control groups, all showing statistical difference ($P < 0.05$) (Table 2).

Table 2
Comparison of migration and proliferation inhibitor gene expression levels as well as contents of epithelial and stromal transforming gene and proliferation related proteins in each group.

Group	*n*	Inhibitor gene expression levels					Transforming gene and proliferation related proteins			
		CD44v6	MMP9	DEC-1	IKK16	MBP-1	β-catenin	Vimentin	E2F1	Bmi-1
Cancerous group	58	3.27 ± 0.42	1.89 ± 0.13	112.38 ± 11.36	73.73 ± 5.86	25.73 ± 1.83	1.59 ± 0.22	4.05 ± 0.48	101.36 ± 9.37	265.31 ± 23.70
Non cancerous group	61	2.23 ± 0.22*	1.17 ± 0.10*	194.34 ± 14.68*	109.87 ± 9.85*	47.27 ± 2.76*	2.87 ± 0.21*	2.08 ± 0.23*	194.64 ± 11.52*	207.42 ± 24.51*
Control group	60	2.17 ± 0.16*	1.98 ± 0.17*	223.48 ± 22.47*	148.78 ± 15.59*	58.38 ± 5.47*	3.09 ± 0.23*	1.97 ± 0.31*	212.68 ± 19.56*	112.86 ± 12.53*

Note: Compared with cancerous group, *$P < 0.05$.

4. Discussion

HPV infection is related with age. Positive young patients may sometimes accept unnecessary colposcopy, biopsy or even over-treatment. High-risk long-term infection of HPV can easily increase the incidence of cervical intraepithelial neoplasia and cervical cancer. HPV is a non-enveloped DNA virus, and has a very strict tissue characteristics, which mainly attacks issues like the skin and the squamous epithelium. According to the HPV subtypes carcinogenic risk level by the World Health Organization, HPV can be divided into two types of low-risk and high-risk. (1) Low-risk is mainly caused by the external genital and anal exocrine warts lesions and low cervical intraepithelial neoplasia. (2) High-risk: in addition to causing genital warts, the more important is the cause of genital cancer, cervical cancer and a high degree of cervical intraepithelial neoplasia. High-risk HPV plays an important role in cervical squamous cell carcinoma, which is closely related to the occurrence of cervical squamous cell carcinoma. The study was found that the positive rate of high-risk HPV type of cervical cancer can reach 50%–90%. The HPV viral genome has many partition ways, of which genetic structure can be divided into three parts: early region (E) and late region (L), and long control region (LCR) [7,8]. The early proteins encoded by E1-6 and E7 are involved in the transcriptional regulation of DNA and the transcriptional regulation of DNA and cell transformation. The occurrence of cervical cancer associated with oncogene activation, tumor suppressor gene inactivation and immunomodulatory mechanism of imbalance caused cell proliferation and apoptosis abnormal changes. HPV E6/E7m RNA is a viral oncogene that binds to the tumor suppressor genes P53 and Rb of the host cell, resulting in cancerous cell cycle control abnormalities. A key factor in the progression of malignant progression of cervical lesions is E6E7 overexpression, and many investigators have suggested that E6E7m RNA, which reflects its transcriptional activity during E6/E7 oncoprotein expression, may be more predictive of disease progression. As the role of E6 and E7 oncoprotein in the carcinogenesis of cervical cancer becomes more and more clear, many researchers have suggested that E6/E7 mRNA expression in E6 and E7 may be more predictive of disease progression. Among the TRAF members, the strongest NF-κB-mediated downstream of CD40 was TRAF6. TRAF6 may be the main mediator of CD40 signaling, and it is not clear that TRAF6 acts on some transcription factor and kinase in the CD40 signaling pathway. Within the cytoplasm of the CD40 there is a site that binds to the framework protein TRAF6. From the linear structure, the distal portion of the cytoplasmic portion of CD40 has a TRAF6 binding site. However, other studies suggest that TRAF6 is not involved in the NF-κB signaling pathway, and the activation of NF-κB in the cells is not affected by the removal of the TRAF6 binding site of CD40. C-Flip is a protein that is very similar to v-Flip. C-Flip can compete with Caspase-8, Caspase-10 and/or FADD by blocking the signal transduction of Caspase-8, Caspase-10 activation and DISC formation through two N-terminal DEDs, leading to the inability to continue Caspases Cascade reaction. It leads to TRAIL-R and other cell death receptor-mediated apoptosis signal transduction block, and thus inhibits the role of apoptosis. C-FLIP is a kind of apoptosis-inhibitory protein found in recent years, containing death effect domain, which can inhibit Fas, DR3, TRAILR-mediated apoptosis. It included to forms, namely C-FLIP-L and c-FLIP-S, respectively. Among them, C-FLIP-L is an important factor affecting drug resistance of tumor cells [9–11]. There was no significant difference in the relative expression of E6 and E7 mRNA of HPV-18 in the non-cancerous group and the control group ($P > 0.05$). In cancerous group, expression of E6 and E7 of HPV-18 were all higher than those in non-cancerous group and control group ($P < 0.05$), which showed significant difference. It indicated that HPV-18 is relevant to the occurrence of cervical cancer, and the increase of relative expression has positive correlation with the occurrence of cervical cancer. The contents of TRAF6 and c-FLIP had no significant differences in non cancerous and control groups ($P > 0.05$). The contents of invasion genes TRAF6 and c-FLIP in cancerous group were all significantly higher than non cancerous and control groups ($P < 0.05$), which indicated that invasion genes in cervical issue plays a role on tissue cells, leading to variability of growth of cervical tissue cell and increase of invasion, which is the influencing factor of the incidence of cervical cancer.

CD44v6 is the most important variant of CD44, belonging to the tumor metastasis-related genes. The encoded protein can promote tumor cells and extracellular matrix components with each other, and then through a variety of transmembrane signaling pathways to promote cell invasion and migration. MMP9 is an important member of the matrix metalloproteinases family, which has hydrolysis on a variety of compositions of extracellular matrix and the basement membrane. Loss of extracellular matrix and basement membrane components creates favorable conditions for tumor cell migration. DEC-1 is widely expressed in many normal tissues and is a kind of transcription factor containing basic helix-loop-helix domain. It regulates the expression of related genes by binding Ebox cis-acting element. Related studies have shown that DEC-1 and chronic and acute hypoxia-related, can regulate cell proliferation and differentiation, and participate in the induction of apoptosis, which has a very important role in the regulation of physiological cycle and tumor occurrence. NF-kB plays a very important role in the occurrence and invasion of glioma and the resistance to chemoradiotherapy. It is mainly protein kinase (IKK) that regulated NF-kB activity, and IKK-16 is one of the IKK-specific inhibitors [12–14]. The major component of IKK is a large protein kinase complex that is activated by NF-κB. The human c-myc promoter binding protein (MBP-1) is located on the human chromosome 1p35-pter, and MBP-1 has no enzymatic activity and is located predominantly in the nucleus. P2 plays an important role in normal cell proliferation, initiating 75%–90% of human c-Myc mRNA. MBP-1 can bind upstream of the transcription initiation site of P2 promoter, and negatively regulates c-Myc. MBP-1 is not only dependent on the expression of c-Myc, but can be linked to the promoter of COX-2 and down-regulated the expression of COX-2. MBP-1 up-regulates miR-29b and inhibits the growth of tumors by inhibiting the expression of Mcl-1, MMP-2 and collagen [15,16]. There was no significant difference in the expression of CD44v6 and MMP9 mRNA in the comparison of the non-cancerous group and the control group ($P > 0.05$). The mRNA levels of CD44v6 and MMP-9 in cervical cancer group were significantly higher than those in non-cancerous group and control group ($P < 0.05$), which indicated that the migration gene of cervical cancer was highly expressed, and the variation of cell migration ability was changed, thus promoting cervical cancer cell migration and distant metastasis. There was no significant difference in the content of proliferation inhibitor genes (DEC-1, IKK16, MBP-1)

between the non-cancerous group and the control group ($P > 0.05$). The proliferation inhibitor genes DEC-1, IKK16, MBP-1 in cervical cancer group were significantly lower than those in non-cancerous group and control group ($P < 0.05$), indicating that the proliferative ability of cervical cancer tissue was affected and the abnormal proliferation was not restricted, leading to cancer cell variation and influencing the incidence of cervical cancer.

Epithelial-mesenchymal transition (EMT) is an important process in which cells obtain kinetic and invasive properties. In the process of EMT, epithelial cells are transformed into mesenchymal cells through specific procedures and acquire strong invasion and metastasis. TGF-β is an upstream regulatory molecule that influences the process of EMT, and NF-kB and Par-4 increase the expression of N-cadherin and Vimentin. Meanwhile, the expression of E-cadherin and β-catenin decreased, the polarity of E-cadherin decreased and the kinetic energy was enhanced, which was more likely to local infiltration and distant metastasis. The E2Fs transcription factor family regulates gene expression and DNA replication associated with cell cycle progression. E2F1 is one of its members, but also has a unique pro-apoptotic effect. It is suggested that E2F1 may play an apoptotic role by enhancing the sensitivity of cells to apoptotic stimuli, inhibiting the activation of anti-apoptotic signaling pathways, activating p53-independent or p53-dependent apoptotic signaling pathways. Human Bmi-1 gene located in the short arm of chromosome 10, 13 region, and its structure contains 10 exons and 10 introns. A protein containing 326 amino acids is encoded by an open reading frame containing a helix-turn-helix-turn domain located in the central conserved DNA-binding domain and a loop-finger domain located at the N-terminus, which both contribute to tumor formation and cell transformation. The present study has confirmed that Bmi-1 in a variety of tumors was high expression, and the occurrence and development of cancer, but the relationship between prognosis is not mentioned in the text. Recently, some studies have confirmed that Bmi-1 directly involved in tumor development and invasion and metastasis. Bmi-1 plays an important role in mammalian, hematopoietic, neurological and skeletal development during embryonic development [17,18]. Bmi-1 cells, which are highly expressed in tumor cells, are considered to be "cancer stem cells" in tumors during tumor development and progression. It is found that Bmi-1 gene paves a new way for the tumor occurrence mechanism, the clinical pathology and prognosis. The mRNA levels of β-catenin and Vimentin in the non cancerous group and the control group were not statistically different ($P > 0.05$). The mRNA levels of β-catenin and Vimentin in cervical cancer group were significantly lower than those in non cancerous group and control group ($P < 0.05$), indicating that the decrease of epithelial marker expression was related to the occurrence of cervical cancer. There was no significant difference in the content of E2F1 and Bmi-1 between the non cancerous group and the control group ($P > 0.05$). The level of Bmi-1 in cervical carcinoma was significantly lower than that in non cancerous group and control group ($P < 0.05$), which indicated that the expression of E2F1 in cervical cancer was reduced, leading to cell proliferation of a series of related gene transcription activation, suggesting that the occurrence of cervical cancer and cell proliferation protein were relevant.

According to the above research, we found that the expression of HPV mRNA increased with the development of cervical cancer, and the expression level of HPV mRNA was also positively correlated with the development of cervical cancer. Through the detection of these indicators, it provides an important clinical guidance for the development of cervical cancer monitoring and treatment.

Conflict of interest statement

We declare that we have no conflict of interest.

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